Integrated immunogenomics in the chicken - Mapping innate immune disease resistance genes

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Integrated Immunogenomics in the Chicken: Deciphering the Immune Response to Identify Disease Resistance Genes

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Abstract: Resistance to infection takes place at many levels, and involves both non-specific and specific immune mechanisms. The chicken has a different repertoire of immune genes, molecules, cells and organs compared to mammals. To understand the role of any disease resistance gene(s), it is therefore important to understand these different repertoires, and the bird's response to a particular pathogen. Our studies focus on the innate immune response, as responses of macrophages from inbred lines of chickens, and heterophils from commercial birds, correlate with resistance or susceptibility to *Salmonella* infection with a variety of *Salmonella* serovars and infection models. To map disease resistance genes, we are using a combination of expression quantitative trait loci (eQTLs) from microarray studies, allied with whole genome SNP arrays (WGA) and a candidate gene approach. There are over 500 human genes with the Gene Ontology term "innate immunity." We have identified over 400 of these genes in the chicken genome, and are actively identifying informative SNPs in them. The segregation of 6,000 WGA SNPs across all of our inbred lines was also assessed, which should yield approximately 900 informative SNPs for a cross between any two lines. The initial focus of these studies is on mapping resistance genes in our inbred lines, but the studies will be extended to commercial flocks.

INTRODUCTION

The availability of genome sequences, not only for pathogens, but also now for the chicken host [1] represents a major shift in our ability to understand host-pathogen interactions. In previous work, we have shown that chickens differ greatly in their susceptibility to a wide range of diseases and vaccination response [2,3]. Our focus is now on understanding the role of cytokines and chemokines in the avian immune response [4], including their potential to act as vaccine adjuvants, and to use the new resources available to map disease resistance genes (e.g. see [5,6]).

The immune system Th1-Th2 paradigm is well established in mammals. Th1 responses control inflammatory reactions to viruses and other intracellular pathogens, and Th2 responses to helminthic worm infections, extracellular pathogens and allergens. Until recently, there was limited information as to whether this paradigm applied to non-mammalian species, such as birds. Cytokines control immune responses and drive them towards Th1 or Th2 responses, and the recent cloning of the first non-mammalian Th2-controlling cytokines [7] means that this question of fundamental importance to understanding the evolution of the immune response can now be addressed. Using these reagents, we demonstrated, for the first time, that this paradigm also extends to chickens [8], in that infections with intracellular pathogens result in a Th1-dominated immune response and infections with extracellular pathogens in a Th2-dominated response. Further, analysis of chicken expression sequence tags (ESTs) and genome sequences has allowed the chicken's full repertoire of cytokines and chemokines and other genes of the avian immune system to be catalogued [1,4]. The results correlate with certain differences in the biology of the chicken compared to biomedical species. For example, chickens lack functional eosinophils, and they also lack IgE, IL-5 and the eotaxins, all molecules crucial for the function of eosinophils in mammals. Chickens also lack lymph nodes, and knockout mouse models have shown that the development of secondary lymphoid organs, such as lymph nodes, is dependent on expression of the lymphotoxin genes. The chicken lacks the genes for lymphotoxins and their receptors.

In recent years, we have played a major role in developing new tools and resources for the analysis of complex traits, such as disease resistance in poultry. This started with the creation of genetic linkage maps of the chicken and recently maps based on single nucleotide polymorphisms or SNPs [6]. These tools have been used to map hundreds of QTL for a wide range of traits (e.g. [5], and summarised in the genetic variation database). The chicken EST project funded by the Biotechnology and Biological Sciences Research Council (BBSRC) played a major part not only in gene discovery but also in annotation of the genome [1]. We have used these ESTs to build high-density gene expression microarrays [9] and recently in collaboration with Affymetrix and others, gene chips have been developed for all known chicken genes (http://www.affymetrix.com/support/technical/byproduct.affx?product=chicken).

Demonstration of the crucial importance of the innate immune response is one of the major advances in immunology of the past few years. Rather than just being an ancestral simple immune response, it is now obvious that the innate immune response both provides an important initial response to pathogens that can limit or even prevent infection, and crucially determines the course of an adaptive immune response, and hence immunological memory (i.e. the ability to respond to future infection with the same pathogen - the basis of vaccination).

There are polymorphisms in the genes controlling innate responses in mammals, which affect function (e.g. in the *TLR4* gene between He/J and He/N mice). Previously we have concentrated on the chicken chemokine gene families and their receptors [4], which are central to controlling the recruitment of immune cells of differing types to sites of infection, the Toll-like receptors ([10] and Burt et al, unpublished data), which are fundamental to the initial recognition of pathogens, and on the defensins [11], whose products are directly harmful to bacteria. Investigating these genes it rapidly became apparent that all of these have been subject to very rapid and distinct evolution since mammals and chickens diverged, both in the sequences of the individual genes, with polymorphisms between the different inbred lines for some genes and in the duplications and chromosomal movements which have taken place in these gene families. This is probably due to strong selective pressures on the genes, arising from the need to develop better immune responses, or to prevent pathogens evading or manipulating the immune response by mimicry of these genes.

There is also evidence that different lines of chickens differ in their innate immune responses to pathogen challenge (particularly the enteric pathogen *Salmonella*), and that this correlates with resistance to infection with those pathogens [12-14]. It therefore seems reasonable to suppose that this differential response is under genetic control, and that similar mechanisms may in part explain the different resistance profiles of the Institute for Animal Health (IAH) inbred lines (see Table 1) of chickens to all the pathogens so far tested (viral, bacterial and protozoan parasites) [2,3].

The identification of innate immune resistance mechanisms also has strategic industrial relevance. The UK poultry industry faces numerous challenges in order to remain sustainable. These include the imminent move to more extensive rearing systems; the withdrawal of prophylactic and many therapeutic antibiotics, and other drugs such as anti-coccidials; and resistance and residue problems with anti-helminthics. These challenges will all have an impact on poultry health. It is important that poultry breeders are able to select for genetic improvement in performance when birds are reared in such environments, and one obvious phenotype would be "robustness." Improved innate resistance would contribute to robustness and would be a general phenomenon, as by its very nature the innate immune response does not differentiate between different pathogens, but rather recognises and responds to pathogen-associated molecular patterns that are conserved across pathogen classes.

The recent availability of the chicken genome marks a quantum shift in our ability to understand the general biology of this important model and commercial organism, and its immune response to infection in particular. Before the genome era, genes involved in immune function have not always been easy to identify, as they are under heavy selective pressure due to the ongoing host-pathogen "arms-race" and thus have limited amino acid identity to their mammalian orthologues. However, many of the genes related to innate immunity (including those encoding defensins, chemokines and their receptors, pro-inflammatory cytokines, the type I interferons (IFNs), natural killer (NK) cell receptors and Toll-like receptors) have already been cloned and sequenced, and the availability of the chicken genome sequence and some comparative breed data from the Beijing Genomics Institute [6] means that additional genes can readily be identified in the chicken by comparison with their mammalian orthologues.

Table 1: Disease resistance of IAH inbred lines of chickens.

	Protozoan parasites[a]							Enteric bacteria[b]						Viruses[c]		
Line[d]	Ea	Ep	En	Ema	Emi	Eb	Et	St	Sg	Se	Sp	St col	C col	IBV	IBDV	MDV
BrL	M	S	S	M	S	M	M							R	S	M
Wl	S	R	R	R	R	R	S	R	R	R	R			M	M	M
15I	S	S	S	S	S	S	R	S	S	S	S			S	R	M
7_2	S	S	R	M	S	S	R	S	S	S	S			S	R	S
6_1	S	S	R	S	S	S	R	R	R	R	R	R	R	M	R	R
C	R	R	R	R	S	S	S	S	S	S	S			R	R	S
N	M	R	R	M	S	R	R	R	R	R	R	S	S	M	S	R
0	R	S	M	M	S	R	R	S	S	S	S				R	R
P															R	S
Sykes RIR															R	S

R = resistant; M = intermediate; S = susceptible.
[a]Ea = *Eimeria acervulina*; Ep = E. *praecox*; En = E. *necatrix*; Ema = E. *maxima*; Emi = E. *mitis*; Eb = E. *brunetti*; Et = E. *tenella*.
[b]St = *Salmonella* Typhimurium; Sg = S. Gallinarum; Se = S. Enteritidis; Sp = S. Pullorum; St col = S. Typhimurium colonisation; C col = *Campylobacter jejuni* colonisation.
[c]IBV = infectious bronchitis virus; IBDV = infectious bursal disease virus; MDV = Marek's disease virus.
[d]BrL = brown leghorn; Wl = Wellcome line; RIR = Rhode Island red.

Materials and Methods

Genomic analysis and identification of innate immune function genes

All genome analysis for identification of chicken innate immune genes was based on Ensembl release 46.2d which is assembly WASHUC2 at http://genome.wustl.edu/genome.cgi?GENOME=Gallus%20gallus. Where gene orthologues were not already identified as such in Ensembl, it and the UCSC genome browser were used extensively for examination of syntenic context of newly identified genes. Chicken genes were considered to have conserved synteny if flanked by at least three recognisable orthologues of the same genes as the orthologous human genes, with the same arrangement and relative orientations.

SNP identification

Primers were designed for PCR amplification and sequencing of portions (800 to 1,000 base pairs) of innate immune candidate genes in a number of birds of each line of interest (lines 6_1 and N). PCR was performed by standard methods and sequencing carried out on a Beckman Coulter CEQ 8000 sequencer. The area of each gene to be sequenced was generally selected based on the presence of a number of SNPs already shown in Ensembl sequence. This denoted that there was likely to be a good level of variation in that area and thus an increased possibility of an informative SNP between our lines. These selected areas were mainly intronic or promoter areas as exons usually contain a much lower level of genetic variation. Sequences were compared between lines using CodonCode Aligner software. Informative SNPs were selected as ones that were different between the two lines and showed no heterozygosity within either line. If no informative SNP was identified in a selected area of a candidate gene, another area of that gene was selected for further sequencing and possible informative SNP detection.

Whole genome analysis using the Illumina beadstation

Four oligonucleotide pools (OPAs) made available by our collaborators (Aviagen and USDA) were screened for SNPs that are fully informative between lines 61 and N for QTL mapping studies, which are polymorphic between lines yet monomorphic within a line. Genomic DNA was extracted from the blood of 10 birds from each of the chicken lines. Whole genome genotyping was performed on 250 ng of DNA for each of the four OPAs (custom 1536 SNP panels, 96-sample Sentrix array matrix) using the Illumina Golden Gate genotyping platform. Automatic clustering of the samples and genotype calling was done with the Illumina BeadStudio software (version 3.0.27) and each locus confirmed independently by two investigators. BeadStudio was also used to assess each assay for Genetrain score, heterozygosity, cluster separation and line-specific homozygosity. This software integrates a genotype call score to give weighted genotype calls. Only assays with a high call rate and reproducibility were selected.

Results

A search of the human Gene Ontology database with the term "innate immunity" identified approximately 510 genes known to have a role in innate immune responses. Of these, we have identified around 480 in the chicken genome. In part, the difference in numbers can be explained by the fact that in general, although chickens have the same multigene families of innate immune response genes as mammals (e.g. TLRs, defensins, proinflammatory chemokines, etc.), there are fewer members of these families in chickens than in mammals.

To date, we have concentrated on identifying SNPs between lines 61 and N in a study on disease resistance (specifically resistance to *Salmonella* and *Campylobacter* gut colonisation), focusing on some of the better characterised "headline" genes (see Table 2), 99 in all. Altogether, 71 informative SNPs (i.e. SNPs that segregate absolutely between the two lines) in 36 genes have been identified. There are also 20 other genes in which SNPs were identified, but these were heterozygous within one or both lines, and thus not usable in mapping studies. Strikingly, we have identified very few SNPs (an informative SNP in TLR 1/6/10 gene 1, and uninformative SNPs in the TLR2 type2, TLR3 and TLR5 genes) in the TLR genes, having sequenced several kilobases of these genes, including promoter regions, exons and introns. In contrast, we readily find SNPs in genes encoding signalling molecules downstream of the TLRs. Of the 71 SNPs, 49, covering all 36 genes, will be incorporated into the final SNP panel for line 6_1 and line N comparisons.

Table 2: Candidate gene approach – innate immune gene panel. Rs = receptors.

TLR pathway:	Interleukins:	Chemokines:	Chemokine Rs:	Defensins:	Other AMPs:
Tollip	*IL1B*	*XCL1*	*XCR1*	*AvBD1*	*LEAP2*
IRAK-4	*IL2*	*CCLi1*	*CCRa*	*AvBD2*	
Mal/TIRAP	*IL3*	***CCLi2***	*CCRb*	*AvBD3*	Other PRRs:
MyD88	***IL4***	*CCLi3*	***CXCR1***	*AvBD4*	*NOD1*
TRAF6	***IL5***	*CCLi4*		*AvBD5*	*MDA5*
MAP3K7IP1	***IL6***	*CCLi5*	TNFSF:	*AvBD6*	
MAP3K7IP2	*IL7*	*CCLi6*	*TNFSF4 (OX40L)*	***AvBD7***	IL Rs:
MAP3K7	*IL9*	*CCLi7*	***TNFSF5 (CD40L)***	*AvBD8*	***IL1R1***
TLR1/6/10 gene 1	***IL10***	*CCLi8*	*TNFSF6 (FASL)*	*AvBD9*	***IL1RL2***
TLR1/6/10 gene 2	***IL12A***	*CCLi9*	***TNFSF8 (CD30L)***	*AvBD10*	***GP130***
TLR2 type 1	*IL12B*	*CCLi10*	***TNFSF10 (TRAIL)***	*AvBD11*	
TLR2 type 2	***IL13***	***CXCLi1***	*TNFSF11 (RANKL)*	*AvBD12*	Other genes:
TLR3	***IL15***	*CXCLi2*	*TNFSF13B (BAFF)*	***AvBD13***	*Caspase 1*
TLR4	*IL16*	***CXCLi3***	***TNFSF15 (VEGI)***		*MIF*
TLR5	*IL17A*		*TRAIL-L*		*NRAMP1*
TLR7	***IL17B***				
TLR15	*IL17D*		CSFs:		
TLR21	*IL17F*		*GCSF*		
IKKA	*IL18*		***GMCSF***		
IKKB	***IL19***				
IKBA	*IL21*		TGFs:		
NFKB1	***IL22***		*TGFB2*		
NFKB2	***IL26***		*TGFB3*		

Genes in bold have informative SNPs. Genes underlined had SNPs, but they were heterozygous within lines 6^1 and/or N.

Whole genome analysis

Analysis of this data identified a panel of approximately 900 markers that will be used to identify informative transmissions between lines 6_1 and N (Fig. 1). Furthermore, it was noted during the genotyping of these samples that a number of SNP assays failed for an entire line (Fig. 2). This was thought to result from differential amplification of alleles that contain divergent probe sequence between the lines. Subsequent sequencing of the regions flanking the assay locus proved this to be the case, resulting in identification of line-specific variation at this site (Fig. 3). An additional set of SNP assays for these "allelic dropout" regions has been incorporated into the final SNP panel for line 6_1 and line N comparisons.

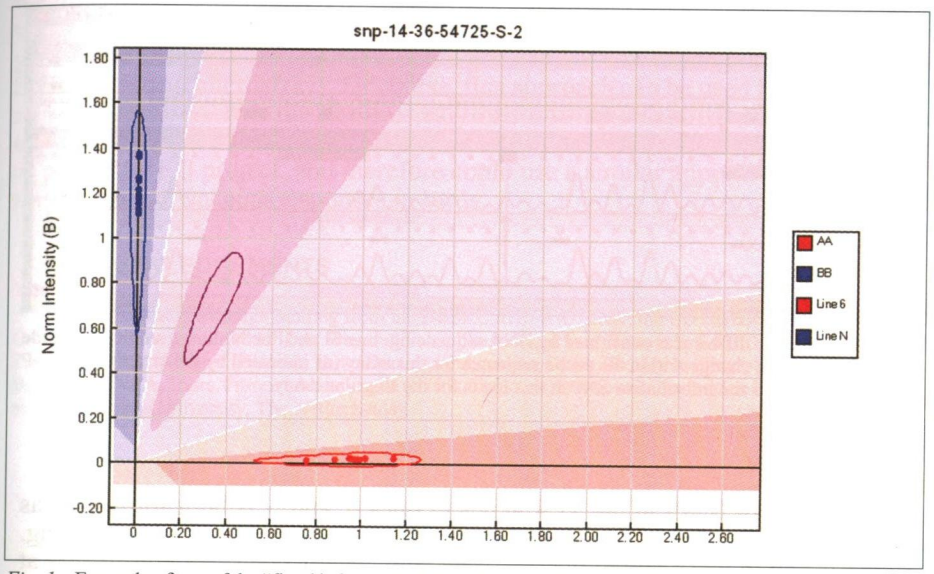

Fig. 1: Example of one of the "fixed informative" line 61/line N SNPs identified in the screening of a chicken 3072 SNP panel (Cartesian Plot).
Line 61 is a "GG" genotype whereas line N is "AA" for this locus. These genotypes carry a high call score, indicating this assay has a good call rate and accuracy.

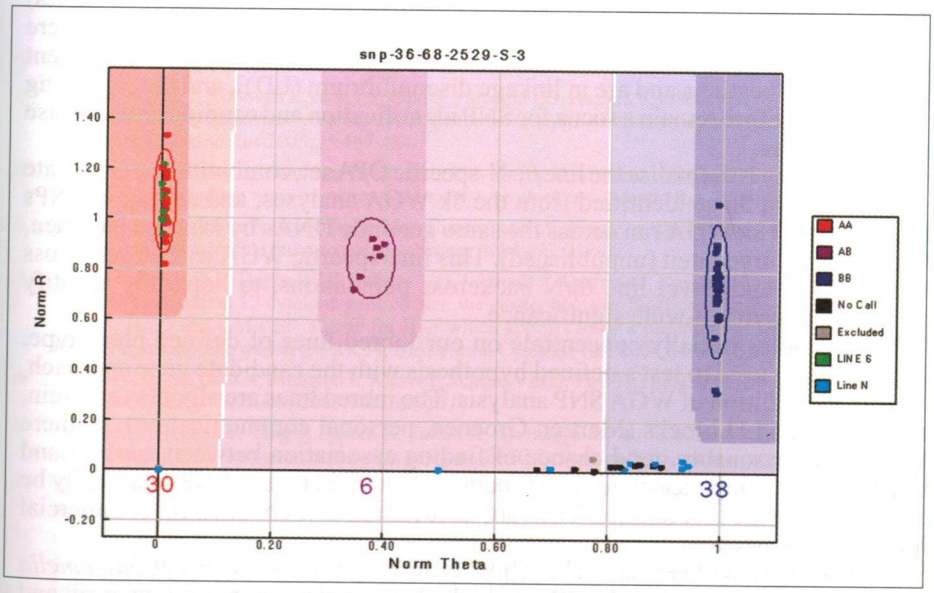

Fig. 2: Allelic dropout – line 6_1 calls as an "AA" genotype whereas line N fails to amplify for this assay, as a result of line-specific SNPs in the probe sequence (Polar Plot). Note that other birds tested are a mix of heterozygote and homozygote at this locus, indicating that the assay is not failing.

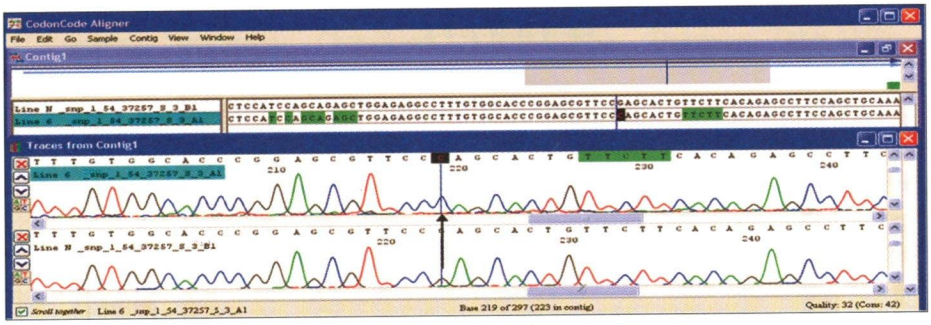

Fig. 3: Allele-specific differences confirmed by DNA sequencing. line 61 and line N show a single nucleotide polymorphism change within the probe sequence for this assay (as indicated by the arrow). This SNP will now act as the informative SNP at this locus for the Illumina assay.

Discussion

The paucity of SNPs in the TLR genes in lines 6_1 and N is both surprising, as SNPs in TLR genes have been described for other chicken lines, and interesting. It suggests that in these inbred lines, at least, any genetic control is presumably at the level of the genes encoding signalling molecules downstream of the TLRs (or potentially at the level of the genes that encode the molecules that control TLR expression). This hypothesis is supported by the fact that numerous "read-out" genes of the induced innate response (e.g. pro-inflammatory cytokines and chemokines) are differentially expressed between our two lines. It is highly unlikely that there are multiple SNPs in each of these "read-out" genes [which have completely different chromosomal locations and are in linkage disequilibrium (LD)], and the signalling molecules therefore remain a focus for SNP identification and candidates as disease resistance genes.

Our next step is to finalise the line 6_1-N-specific OPA set, combining the candidate gene SNPs with those identified from the 6k WGA analyses, and adding in SNPs from a further 18k WGA run across the same genomic DNAs by Martien Groenen, Wageningen Universiteit (unpublished). This line-specific WGA will be put across both archived and novel line 6_1/N backcross populations, to hopefully identify markers with genome-wide significance.

These studies initially concentrate on our inbred lines of defined phenotype. They are being used to test a defined hypothesis with the candidate gene approach, but with the addition of WGA SNP analysis. The inbred lines are all of layer origin, and have large LD blocks (Martien Groenen, personal communication), so there should be a reasonably good chance of finding association between markers and the phenotype being measured. The function of candidate gene SNPs can easily be tested between the inbred lines. Then any association in more outbred, commercial flocks will be assessed.

Microarrays have been run for both lines following infection with both *Salmonella* and *Campylobacter*, using the "Roslin" chicken whole genome microarray, and analysis to identify potential eQTLs is ongoing.

Our overall approach is primarily to understand immune responses to infection in the chicken, an animal that is not just a mouse with feathers. Birds have a different repertoire of immune organs, cells, molecules and genes than mammals, and therefore

we need to understand the bird's immune response to disease so as to understand which genes might be important in that response. The aim is to link phenotype, genotype and eQTLs, using a candidate gene approach, whole genome scans with SNP panels and microarrays. Of course, this approach can be used for any disease-relevant cross, not just the one currently under study. We have also characterised all of the IAH inbred lines for their vaccine responsiveness, at least to killed vaccines, as part of an EU project, and therefore could use a similar approach to investigate the genetics of vaccine responses in birds.

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